JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2005, p. 472–475 Vol. 43, No. 1
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.1.472–475.2005

Clonal Spread of a *Clostridium difficile* Strain with a Complete Set of Toxin A, Toxin B, and Binary Toxin Genes among Polish Patients with *Clostridium difficile*-Associated Diarrhea

Hanna Pituch,[1]* Deborah Kreft,[2] Piotr Obuch-Woszczatyński,[1] Dorota Wultańska,[1] Felicja Meisel-Mikołajczyk,[1] Mirosław Łuczak,[1] and Alex van Belkum[2]

Department of Medical Microbiology, The Medical University of Warsaw, Warsaw, Poland[1]; Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Erasmus University Medical Centre Rotterdam, Rotterdam, The Netherlands[2]

Received 14 June 2004/Returned for modification 13 August 2004/Accepted 1 September 2004

Clinically relevant *Clostridium difficile* strains usually produce toxins A and B. Some *C. difficile* strains can produce an additional binary toxin. We report clonality among five strains carrying all toxin genes from Polish patients with *C. difficile*-associated diarrhea. In another strain, possible recombination between binary toxin genes is documented.

Clostridium difficile is the main etiological agent of nosocomial diarrhea (3, 28). Clinically important *C. difficile* strains usually produce two toxins: toxin A (TcdA), which is an enterotoxin, and toxin B (TcdB), a cytotoxin (3, 5). Recently, toxin A-negative, toxin B-positive *C. difficile* strains have been recovered from patients with symptomatic illnesses from multiple countries (2, 11, 14, 17, 18, 27). In addition to TcdA and TcdB, some *C. difficile* strains can produce a third toxin, called the binary toxin (CDT) (16, 20). Most of the binary toxin-positive *C. difficile* strains produce TcdA and TcdB as well (23), but exceptions to this rule have been documented (7).

The etiological role of TcdA and TcdB in the pathogenesis of *C. difficile*-associated diarrhea (CDAD) has been established, but the significance of CDT in intestinal disorders has not yet been fully elucidated. CDT is related to the clostridial binary toxins, including the iota toxin produced by *Clostridium perfringens* type E, the toxin produced by *Clostridium spiroforme*, and the C2 toxin from *Clostridium botulinum* types C and D (1, 10, 15, 19, 25). TcdA and TcdB of *C. difficile* are encoded by two genes, *tcdA* and *tcdB*, located within the 19.6-kb pathogenicity island PaLoc (4). Different studies have described polymorphism in the *C. difficile* toxin A and B genes (21, 22, 24). CDT is encoded by two other genes, *cdtA* and *cdtB*, both located outside the PaLoc (22, 23). The genes encode two unlinked protein chains (CDTa and CDTb). The 45-kDa enzymatic component CDTa catalyses the ADP-ribosylation reaction of monomeric actin and induces disorganization of the cytoskeleton. The binding component CDTb, about 100 kDa in size, recognizes a cell surface receptor which facilitates translocation of CDTa (1, 9, 16). These combined activities suggest that the binary toxin could effectively contribute to pathogenicity, although this has not been explicitly proven to date. The first binary toxin-positive strain (CD196) was recognized among clinical isolates of *C. difficile* 15 years ago (20). The CDT locus from this strain was cloned and sequenced (GenBank accession number L76081) (16). *C. difficile* CCUG 20309 was the first binary toxin-positive strain producing TcdB but not TcdA. Also, the binary toxin genes for this strain were sequenced (GenBank accession number AF271719), showing 99.6% homology with the CD196 binary toxin genes (6). A *cdtA* gene with 98.3% nucleotide identity and 99.5% amino acid similarity with CD196 was reported for an isolate from a horse (5).

The aim of the present study was to detect and examine the genetic relationship between strains possessing binary toxin genes. The study was performed among Polish patients with CDAD.

Isolates of *C. difficile* ($n = 140$) included in this study were isolated between 1999 and 2003 from as many patients hospitalized in a large university hospital and a distant pediatric hospital. Patients suffering from CDAD were those individuals who produced more than three liquid stools within 48 h, had an antibiotic therapy in their recent medical history, and had a hospitalization period of more than 5 days. Stool cultures should be negative for *Salmonella* spp., *Shigella* spp., pathogenic *Escherichia coli* strains, rotaviruses, and intestinal parasites. Among the *C. difficile* strains, 114 were isolated from adults hospitalized in the university hospital. Patients were nursed in different departments: transplantation ($n = 48$), internal medicine ($n = 19$), general surgery ($n = 22$), orthopedics ($n = 15$), intensive care ($n = 4$), pulmonology ($n = 1$), urology ($n = 2$), dermatology ($n = 1$), and gynecology ($n = 1$). One strain was isolated from an outpatient. A further 26 strains were isolated from children (age range, between 3 and 16 years) suffering from CDAD and being nursed in a separate pediatric hospital. For comparative reasons, we included a toxigenic control strain (VPI 10463) and a nontoxigenic reference strain (NIHBRIGGS 8050) in the cytotoxin assays and TcdA- and TcdB-specific PCR tests. An additional $TcdA^-$ $TcdB^+$ strain (GAI 95601) isolated by H. Kato (Institute of Anaerobic Bacteriology, Gifu University School of Medicine, Gifu, Japan) was used as an internal control for detecting repeated sequences in the *tcdA* gene (12, 17). One reference

* Corresponding author. Mailing address: Chair and Department of Medical Microbiology, The Medical University of Warsaw, 5 Chalubinski Street, 02-004 Warsaw, Poland. Phone: 00478-226282739. Fax: 00478-226282739. E-mail: hanna.pituch@ib.amwaw.edu.pl.

strain, *C. difficile* CCUG 20309, was used as a control for PCR testing for detecting the binary toxin genes *cdtA* and *cdtB*.

All 140 *C. difficile* strains isolated from patients with CDAD included in this study were tested for production of TcdA and TcdB. A single colony was transferred into brain heart infusion broth (Difco, Detroit, Michigan) and grown for 48 h. Supernatants were collected by centrifugation at 3,000 × *g* for 15 min. TcdA was detected by the *C. difficile* toxin A test (Oxoid, Basingstoke, United Kingdom). Additionally, the *C. difficile* TOX A/B test (TechLab, Inc., Blacksburg, Va.) was used for detection of either or both TcdA and TcdB. TcdB was detected by using the McCoy cell line (17). Tenfold serial dilutions of culture filtrate were added in duplicate to McCoy cells and incubated for 24 h. The cytopathic effect (CPE) was observed by inverse microscopy. If this CPE could be neutralized by polyclonal antiserum to *C. difficile* (*C. difficile* TOX-B Test, TechLab, Inc.), the test was considered positive. Albumin was used as a negative control. For detection of nonrepeating sequences in the *tcdA* and *tcdB* genes and repeating sequences in the *tcdA* gene, PCR was performed with specific primer pairs YT28-YT27, YT17-YT18, and NK9-NKV011 as described previously (12, 17, 18). Crude template DNA was prepared by using Genomic DNA PREP-PLUS (A&A Biotechnology, Warsaw, Poland) according to the manufacturer's instructions. For detection of the binary toxin genes, PCR was used as well. Primers designated to amplify regions of *cdtA* and *cdtB* were as follows: cdtA pos, 5′-TGAACCTGGAAAAGGTGATG-3′ (position 507 to 526 in the *cdtA* gene); cdtA rev, 5′-AGGAT TATTTACTGGACCATTTG-3′ (position 882 to 860); cdtB pos, 5′-CTTAATGCAAGTAAATACTGAG-3′ (position 368 to 389 in the *cdtB* gene); cdtB rev, 5′-AACGGATCTCTTGC TTCAGTC-3′ (position 878 to 858). Template nucleic acid (2 μl) was added to 22.5 μl of Supermix (Gibco BRL, Paisley, United Kingdom) and 1 μl of each primer solution (25). PCR products were sequenced bidirectionally without prior purification by cycle sequencing performed commercially at BaseClear (Leiden, The Netherlands). Sequences obtained were aligned and investigated for single nucleotide polymorphisms by use of DNAStar software (Madison, Wis.).

C. difficile binary toxin-positive isolates were cultured for 24 h on Columbia agar. A few colonies were resuspended in 200 μl of lysis buffer, and DNA was isolated as described above. PCR-mediated ribotyping employed consensus primers SP1 and SP2 (5′-TTGTACACACACCGCCCGTCA-3′ and 5′-GGTACCTTAGATGTTTCAGTTC-3′) (13). Ten microliters of DNA was added to a PCR mixture (50 μl) containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin (wt/vol), 0.1% Triton X-100 (vol/vol), 0.2 mM concentrations of each of the four deoxyribonucleotide triphosphates (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom), 1.2 U of Super Taq DNA polymerase (HT Biotechnology, Cambridge, United Kingdom), and 50 pmol of each of the primers. Amplification was performed in a GeneAmp PCR system 9700 cycler (Applied Biosystems, Gouda, The Netherlands) with predenaturation at 94°C for 120 s followed by 40 cycles of 60 s at 94°C, 60 s at 55°C, and 60 s at 74°C. Amplicons were analyzed by electrophoresis on a 0.8% agarose gel for 3 h at 100 V. PCR ribotypes were defined on the basis of a single band position difference in the fingerprints (18, 26).

A total of 142 *C. difficile* strains, including controls, were analyzed by immunoassays and latex tests for detection of TcdA and/or TcdB. In addition, cytotoxicity tests for the specific detection of TcdB were performed. Among these strains, 95 strains were $TcdA^+$ $TcdB^+$, as demonstrated by the *C. difficile* toxin A test, the TOX A/B test, and TcdB-dependent cytotoxicity testing on McCoy cells. Thirty-seven strains were $TcdA^-$ $TcdB^+$, and a CPE was observed after cell line challenge. TcdA could not be detected by the commercial toxin A test. The toxin TOX A/B tests gave positive results for all 37 strains. The remaining 10 strains were $TcdA^-$ $TcdB^-$, as shown by the fact that all tests to detect both toxin TcdA and toxin TcdB were negative. PCR amplification with YT28-YT29 and YT17-YT18 generated products of 630 and 399 bp for the *tcdA* and *tcdB* genes, respectively, for all 95 $TcdA^+$ $TcdB^+$ strains and 37 $TcdA^-$ $TcdB^+$ strains. For the 37 $TcdA^-$ $TcdB^+$ strains, PCR with the NK9-NK011 primer set generated a 700-bp product similar to that obtained for the Japanese GAI 95601 strain. For the 10 $TcdA^-$ $TcdB^-$ strains, PCR did not generate a product, since apparently, the *tcdA* and *tcdB* genes are fully absent.

The presence of the *cdtA* and *cdtB* genes was tested by PCR for the same set of strains. Both *cdtA* and *cdtB* were identified in six strains. All strains harboring *cdtA* and *cdtB* genes also produced TcdA and TcdB. Five $TcdA^+$ $TcdB^+$ and binary toxin-positive strains (118, 2509, 650, 908, and X-1) share identical partial *cdtA* and *cdtB* sequences. Strain 2145 showed deviating sequences (Table 1). The sequences comprised 327 bp of *cdtA* and 451 bp of *cdtB*. The data obtained by PCR ribotyping conformed with the binary toxin gene sequence classification (Table 1). The five identical *C. difficile* strains were isolated at different points in time, with one strain isolated from an outpatient. There was no apparent epidemiological linkage between the individuals. However, we still conclude that endemicity was the case in this hospital, although the source of the infections remains enigmatic. The level of endemicity was apparently different in the two hospitals (6 of 114 versus 0 of 26 binary toxin-positive strains). Similar differences for separate hospitals have been presented before from Paris, France, as well (8).

Strain 2145 shares significant sequence homology with CD196 in the *cdtA* portion; only three mutations were observed. The *cdtB* part, however, is strongly different from both reference sequences. The *cdtB* region of the reference strains is better conserved than the *cdtA* region (0 of 11 mutations versus 4 of 10 mutations; two-sided Fisher exact test, $P = 0.0351$). It has to be emphasized, however, that although they are statistically significant, these figures have to be interpreted with care because of the low number of entries. All Polish isolates can be distinguished from both reference sequences.

C. difficile is commonly isolated from patients with CDAD (28). TcdA and TcdB are the most common virulence factors (3), but the precise relevance of CDT is still a matter of scientific debate (16, 20). In the present study, we identified six clinical *C. difficile* strains with a complete set of binary toxin genes *cdtA* and *cdtB*. Overall, the prevalence as determined in Paris by Goncalves et al. (8) was similar. However, it has to be realized that five out of six Polish strains belong to one type, whereas six types were found among the 17 French isolates. These differences in clonal superstructure affect the genuine

TABLE 1. Survey of PCR ribotypes and partial sequences for the ADP-ribosylating binary toxin genes of various clinical *C. difficile* strains from Poland compared to the reference strains CD196 and CCUG 20309[a]

Strain ID code	Isolation date	Clinical department	PCR type	*cdtA* gene region										*cdtB* gene region										
				23	98	165	169	172	201	217	234	261	318	39	57	58	77	79	124	160	177	218	258	376
CCUG 20309	nr	nr	B	A	G	G	A	T	G	A	G	A	T	C	A	A	G	T	A	G	A	G	A	T
CD196	nr	nr	C	A	G	G	C	T	T	G	G	A	A	C	A	A	G	T	A	G	A	G	A	T
118	01/00	Ortho	A	A	A	A	A	G	C	A	G	A	G	T	A	A	A	G	C	G	C	A	A	A	C
2509	11/01	Trans	A	A	A	A	A	G	C	A	G	A	G	T	A	A	A	G	C	G	C	A	A	A	C
650	05/99	Inter	A	A	A	A	A	G	C	A	G	A	G	T	A	A	A	G	C	G	C	A	A	A	C
908	03/01	Ortho	A	A	A	A	A	G	C	A	G	A	G	T	A	A	A	G	C	G	C	A	A	A	C
X-1	09/01	Outpa	A	A	A	A	A	G	C	A	G	A	G	T	A	A	A	G	C	G	C	A	A	A	C
2145	06/03	Gynec	D	G	G	G	G	C	T	G	G	G	A	T	C	w	A	T	T	G	T	C	A	G	C

[a] All variable sequence positions in the PCR products are stated in this table (e.g. positions 23, 98, 165, etc. in the *cdtA* gene-specific PCR product). The sequences for CCUG 20309 and CD196 were determined by Chang and Song (6) (AF271719) and Perrelle et al (16) (L76081), respectively. The strains derive from Singapore and France. The sequence comparison involves two shorter parts of the *cdtA* and *cdtB* sequences. Within the AF271719 sequence, this comprises a 374-nucleotide-long PCR product between nucleotides 576 and 949 and a 508-nucleotide-long PCR product between nucleotides 1813 and 2322, respectively. nr, not relevant; Ortho, orthopedics; Trans, transplantation; Inter, internal medecine; Outpa, outpatient clinic; Gynec, gynecology; w, A/T. Dates of isolation are in month/year format. Note that the *cdtB* regions of the reference strains are better conserved than the *cdtA* regions (0 of 11 mutations versus 4 of 10 mutations; $P = 0.0351$). Strains 118, 2509, 650, 908, and X-1 share identical *cdtA* and *cdtB* sequences. In addition, the ribotypes are indiscriminate, suggesting clonal spread of this *C. difficile* strain. Strain 2145 shares significant sequence homology with CD196 in the *cdtA* portion (three mutations). The *cdtB* part, however, is strongly different from both reference sequences, possibly suggesting recombination events between different toxin gene sets.

incidence of a particular type of *C. difficile* strain. Strains with a single binary toxin gene were not identified, which is in agreement with data from Stubbs et al. (25). *C. difficile* strains with only the *cdtB* gene were described by Perelle et al. (16). Our partial sequences of *cdtA* and *cdtB* for the six Polish strains clustered into two groups. Stubbs et al. (25) partially sequenced both binary toxin genes of 11 *C. difficile* strains and found three clusters for CDTa and five groups for CDTb. Our data suggest clonal spread of a binary toxin-positive strain in our institution. The recent study performed in Paris (8) failed to document clonality among strains except for those cases where a relapse of the infection was considered likely. Interestingly, strain 2145 shares significant sequence homology with reference strain CD196 in the *cdtA* portion. The *cdtB* part is strongly different from both reference sequences, suggesting the occurrence of recombination events between different toxin gene sets or an enhanced mutability of the CDTb-encoding gene. Although we do not know whether all binary toxin genes are equally well expressed among our strains, in order to define the clinical impact of the binary toxin, prospective clinical studies need to be undertaken.

ACKNOWLEDGMENTS

This work was partially supported by a Concerted Action sponsored by the European Union (CA QLK2-CT-2001-01267).

We acknowledge the support of J. Mainil and C. Duchesnes (University of Liege, Liege, Belgium). We thank Maja Rupnik (Department of Biology, University of Ljubljana, Ljubljana, Slovenia) for *C. difficile* strain 8864 for comparison.

REFERENCES

1. **Aktories, K., and A. Wegner.** 1992. Mechanism of the cytopathic action of actin-ADP-ribosylating toxins. Mol. Microbiol. **6:**2905–2908.
2. **Alfa, M. J., A. Kabani, D. Lyerly, S. Moncrief, L. M. Neville, A. Al-Barrak, G. K. H. Harding, B. Dyck, K. Olekson, and J. M. Embil.** 2000. Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile* responsible for a nosocomial outbreak of *Clostridium difficile*-associated diarrhea. J. Clin. Microbiol. **38:**2706–2714.
3. **Boriello, S. P.** 1998. Pathogenesis of *Clostridium difficile* infection. J. Antimicrob. Chemother. **41**(Suppl. C)**:**13–19.
4. **Braun, V., T. Hundsberger, P. Leukel, M. Sauerborn, and C. von Eichel-Streiber.** 1996. Definition of the single integration site of the pathogenicity locus in *Clostridium difficile*. Gene **181:**29–38.
5. **Braun, V., C. Herholz, B. Straub, B. Choisat, J. Frey, J. Nicolet, and P. Kuhnert.** 2000. Detection of the ADP-ribosyltransferase toxin gene (*cdtA*) and its activity in *Clostridium difficile* isolates from Equidae. FEMS Microbiol. Lett. **184:**29–33.
6. **Chang, S. Y., and K. P. Song.** 2001. ADP-ribosylating binary toxin genes of *Clostridium difficile* strain CCUG 20309. DNA Sequence **12:**115–120.
7. **Geric, B., S. Johnson, D. N. Gerding, M. Grabnar, and M. Rupnik.** 2003. Frequency of binary toxin genes among *Clostridium difficile* strains that do not produce large clostridial toxins. J. Clin. Microbiol. **41:**5227–5232.
8. **Gonçalves, C., D. Decré, F. Barbut, B. Burghoffer, and J.-C. Petit.** 2004. Prevalence and characterization of a binary toxin (actin-specific ADP-ribosyltransferase) from *Clostridium difficile*. J. Clin. Microbiol. **42:**1933–1939.
9. **Gülke, I., G. Pfeifer, J. Liese, M. Fritz, F. Hofmann, K. Aktories, and H. Barth.** 2001. Characterization of the enzymatic component of the ADP-ribosyltransferase toxin CDTa from *Clostridium difficile*. Infect. Immun. **69:**6004–6011.
10. **Hatheway, C. L.** 1990. Toxigenic clostridia. Clin. Microbiol. Rev. **3:**66–98.
11. **Johnson, S., S. P. Sambol, J. S. Brazier, M. Delmée, V. Avesani, M. M. Merrigan, and D. N. Gerding.** 2003. International typing study of toxin A-negative, toxin B-positive *Clostridium difficile* variants. J. Clin. Microbiol. **41:**1543–1547.
12. **Kato, H., N. Kato, S. Katow, T. Muegawa, S. Nakamura, and D. Lyerly.** 1999. Deletions in the repeating sequences of the toxin A gene of toxin A-negative, toxin B-positive *Clostridium difficile* strains. FEMS Microbiol. Lett. **175:**197–203.
13. **Kostman, J. R., T. D. Edlind, J. J. LiPuma, and T. L. Stull.** 1992. Molecular epidemiology of *Pseudomonas cepacia* determined by polymerase chain reaction ribotyping. J. Clin. Microbiol. **30:**2084–2087.
14. **Limaye, A. P., D. K. Turgeon, B. T. Cookson, and T. R. Fritsche.** 2000. Pseudomembranous colitis caused by a toxin A^- B^+ strain of *Clostridium difficile*. J. Clin. Microbiol. **38:**1696–1697.
15. **Perelle, S., M. Gibert, P. Boquet, and M. R. Popoff.** 1993. Characterization of *Clostridium perfringens* iota-toxin genes and expression in *Escherichia coli*. Infect. Immun. **61:**5147–5156.
16. **Perelle, S., M. Gibert, P. Bourlioux, G. Corthier, and M. R. Popoff.** 1997. Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. Infect. Immun. **65:**1402–1407.
17. **Pituch, H., N. van den Braak, W. van Leeuwen, A. van Belkum, G. Martirosian, P. Obuch-Woszczatyński, F. Meisel-Mikołajczyk, and M. £uczak.** 2001. Clonal dissemination of a toxin A-negative/toxin B-positive *Clostridium difficile* strain from patients with antibiotic-associated diarrhoea in Poland. Clin. Microbiol. Infect. **7:**442–446.
18. **Pituch, H., A. van Belkum, N. van den Braak, P. Obuch-Woszczatyński, H. Verbrugh, F. Meisel-Mikołajczyk, and M. £uczak.** 2003. Recent emergence of an epidemic clindamycin-resistant clone of *Clostridium difficile* among Polish patients with *C. difficile*-associated diarrhoea. J. Clin. Microbiol. **41:**4184–4187.
19. **Popoff, M. R., and P. Boquet.** 1988. *Clostridium spiroforme* toxin is a binary toxin which ADP-ribosylates cellular actin. Biochem. Biophys. Res. Commun. **152:**1361–1368.
20. **Popoff, M. R., E. J. Rubin, D. M. Gill, and P. Boquet.** 1988. Actin-specific

ADP-ribosyltransferase produced by a *Clostridium difficile* strain. Infect. Immun. **56:**2299–2306.
21. **Rupnik, M., V. Avesani, M. Janc, C. von Eichel-Streiber, and M. Delmée.** 1998. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. J. Clin. Microbiol. **36:**2240–2247.
22. **Rupnik, M.** 2001. How to detect *Clostridium difficile* variant strains in a routine laboratory. Clin. Microbiol. Infect. **7:**417–420.
23. **Rupnik, M., M. Grabnar, and B. Geric.** 2003. Binary toxin producing *Clostridium difficile* strains. Anaerobe **9:**289–294.
24. **Spigaglia, P., and P. Mastrantonio.** 2002. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. J. Clin. Microbiol. **40:**3470–3475.
25. **Stubbs, S., M. Rupnik, M. Gibert, J. S. Brazier, B. Duerden, and M. Popoff.** 2000. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. FEMS Microbiol. Lett. **186:**307–312.
26. **Stubbs, S. L. J., J. S. Brazier, G. L. O'Neill, and B. I. Duerden.** 1999. PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. J. Clin. Microbiol. **37:**461–463.
27. **van den Berg, R. J., E. C. J. Claas, D. H. Oyib, C. H. W. Klaassen, L. Dijkshoorn, J. S. Brazier, and E. J. Kuijper.** 2004. Characterization of toxin A-negative, toxin B-positive *Clostridium difficile* isolates from outbreaks in different countries by amplified fragment length polymorphism and PCR ribotyping. J. Clin. Microbiol. **42:**1035–1041.
28. **Wilkins, T. D., and D. M. Lyerly.** 2003. *Clostridium difficile* testing: after 20 years, still challenging. J. Clin. Microbiol. **41:**531–534.